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Development of a continuously operated reactor for the limited hydrolysis of whey protein by trypsin

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Abstract

A continuously operated reactor was developed for the limited hydrolysis of whey protein using soluble trypsin. It consisted of a stirred tank followed by a tubular device. A stirred tank was used because it allowes pH control to be achieved more easily. The tubular reactor was applied in order to achieve a high level of conversion without pH control. The tubular reactor was equipped with static mixers for approximating plug-flow behaviour. The behaviour of the reaction system under floating pH in both parts of the reactor was evaluated separately prior to verification of the results in a pilot-plant which consisted of the combination reactor. The operating temperature and pH in the stirred tank were investigated as the main variables of the process. © 1998 Elsevier Science Ltd

Keywords: trypsin, whey protein, infant food, continuous reactor, CSTR, tubular reactor.

Abbreviations: CSTR, continuous stirred tank reactor, NPN, fraction of non-protein nitrogen or soluble protein, TR, tubular reactor, SN-TCA, fraction of soluble (protein) nitrogen in trichloroacetic acid, WPC, whey-protein concentrate.

Nomenclature

c	Substrate concentration in terms of peptide bonds (mol litre^{-1})
E	Enzyme concentration (kg kg^{-1})
pK_a	Acid dissociation exponent
S	Substrate concentration (kg kg^{-1})
t	Time, reactor operating time (min)
T_c	Temperature (°C)
V_R	Reactor volume (m^3)
$\dot{V}$	Flow rate (m^3 min^{-1})
X	Degree of hydrolysis, fraction of peptide bonds cleaved, NPN
x	Molar fraction
x_H	Fraction of hydronium ions formed per fraction of peptide bonds cleaved
Y	Yield of soluble protein, NPN according to the SN-TCA method
β	Buffer capacity
τ	Space time, residence time ($= V_R/\dot{V}$) (min)

Indices

a	Referring to the acid form
b	Referring to the base form
C	Referring to the carboxyl group
f	Referring to final conditions
N	Referring to the amino group
0	Referring to initial conditions

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Introduction

Hypoallergenic infant food commonly contains peptides obtained from readily available natural proteins which are treated with proteolytic enzymes to achieve limited protein hydrolysis, thereby destroying the allergenic epitopes of natural proteins [1]. Processes for manufacturing these peptides commonly use discontin-

uously operated stirred tank reactors in the presence of soluble enzymes such as trypsin. Continuous reactor operation is of major interest because the product is in high demand and there is a need to manufacture a product of constant quality. Both criteria favour continuously operated reactors over batch reaction systems. However, the peculiarities of protein hydrolysis limit the choice of reactor design. Thus, immobilization of enzymes on porous supports is inefficient due to the presence of macromolecular substrates which in addition, lead to support fouling [2]. Ultrafiltration membrane reactors have been operated with limited success for the same reasons [3,4]. Another major characteristic of protein hydrolysis is that substrate conversion is sometimes accompanied by a drastic drop in pH, which may require pH-controlled reactors to be used [5].

A reactor was designed for continuous operation being based on the use of soluble enzymes, and in which most of the pH drop is avoided by pH control. This was achieved with a two stage design, consisting of a continuously operated stirred tank reactor preceeding a tubular reactor. The initial pH and operating temperatures were investigated so that these main operating variables could be set in order to achieve a fraction of soluble protein of at least 60%.

Materials and methods

Partialy demineralized whey-protein concentrate (WPC) with an average protein content of 22% (w/w) and porcine pancreatic trysin, PTN 6·0 S (Novo Nordisk, Denmark) were used. The trypsin preparation contained 10·2 μmol g^{-1} of active enzyme as estimated by active-site titrations according to the method of Chase and Shaw [6]. All experiments were performed at an initial substrate concentration of WPC of 20% (w/w) (S_0 = 0·2). The standard condition with respect to pH was 7·3. The WPC contained an initial fraction of (apparently) soluble protein of 4·8% (Y_0 = 0·048) consisting mainly of soluble peptides as well as ammonia. Trypsin was solubilized in 1 mM HCl containing 5 mM $CaCl_2$ prior to being added or fed to the reaction systems.

The fraction of protein soluble in 13·6% trichloroacetic acid (SN-TCA index) expressed as non-protein nitrogen (NPN) or fraction of soluble protein (Y) was calculated from correlations with base consumption in the case of pH-controlled reactor experiments or determined directly. Details about batch-reactor operation, analytical procedures and the correlation of the SN-TCA index (NPN) with base consumption may be found in a previous report [5]. Protein solutions were treated for 5 min at 90°C prior to starting the reaction by the addition of enzyme solution. In the case of pH-controlled reactions, a solution of 4 M KOH was fed into the reactor automatically in order to achieve constant pH. All experiments were performed with an enzyme–substrate ratio of 0·01 kg kg^{-1}.

For continuous operation a pilot-plant was designed consisting of a tubular reactor (TR) in series with a continuous stirred tank reactor (CSTR) as shown in Fig. 1. The geometry of the CSTR is detailed in the

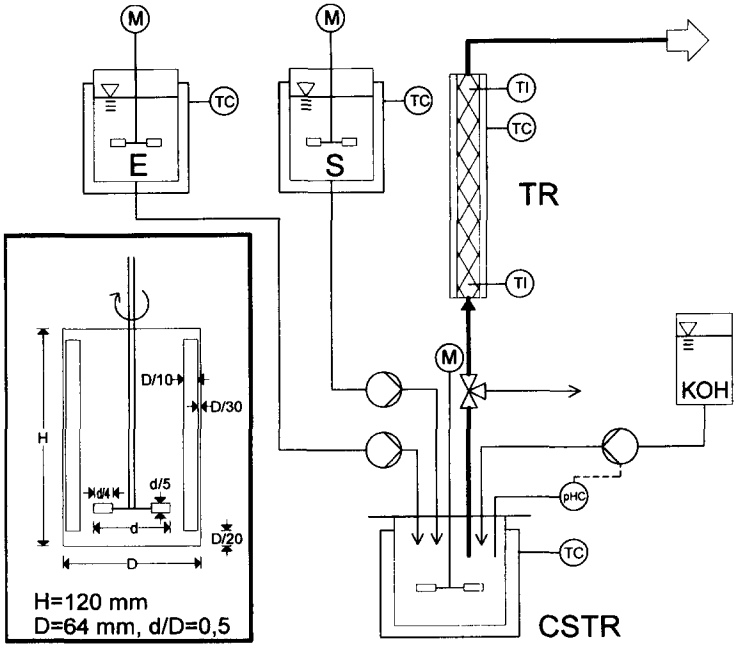

Fig. 1. Flow sheet of the pilot-plant reactor. The insert shows the geometry of the continuous stirred tank reactor (CSTR). The tubular reactor (TR) contains static mixers. Reservoirs for enzyme solution (E), substrate solution (S), and 4 M KOH (KOH) are shown.

insert of Fig. 1. It consisted of a cylindrical tank equipped with a six-bladed Rushton turbine and four baffles. During operation the CSTR was filled completely. A water jacket served for thermostating. The turbine was kept at a constant speed of 1000 min^{-1} by means of a speed-controlled electric motor (model MS, Ismatec SA, Switzerland). The CSTR was fed with both a substrate solution, by means of a peristaltic pump (model IP-4, Ismatec SA, Switzerland), and an enzyme solution, by means of a piston pump (model LKB 2249, Pharmacia, Sweden). The CSTR was equipped with a pH electrode (model HA 405 DXKS8, Ingold Meßtechnik, Switzerland). In conjunction with a titrator/controller (model 614, Metrohm, Switzerland) it served as a pH control by feeding a 4 M KOH with an autoburette (model E535, Metrohm, Switzerland). The tubular reactor, fed by the outlet of the CSTR, consisted of a jacketed glass tube (Quickfit, Germany) with an internal diameter of 40 mm in sections with lengths of 33, 66, and 159 cm, respectively. The sections could be assembled as required. Tubular reactors with effective volumes of 1·82 and 3·04 litres have been used. The tubular reactor was equipped with static mixers (model SMX 39, Sulzer SA, Switzerland) in order to obtain a reactor with low backmixing [7]. The temperature of the tubular reactor was controlled independently of that of the CSTR by means of a thermostat.

Results and discussion

The pH characteristics of the reaction system is of primary importance in a continuously operated reactor. Trypsin exhibits optimal proteolytic activity in the pH range 7–9 and activity decreases rapidly at pH values lower than 6. At alkaline pH, however, trypsin is sensitive to accelerated deactivation by autodigestion. Protein hydrolysis in the pH range of optimum trypsin activity is accompanied by a considerable production of hydronium ions [5] and a reaction without pH control at an initial pH of 7–9 would suffer from a sharp drop in pH. For this reason, a single tubular reactor with single passage would not be the optimum choice due to the inability to control pH.

Analysis of pH shift during protein hydrolysis

The pH profile in reactors operated without pH control may be estimated roughly by applying a simple model. It is assumed that the substrate is devoid of buffering capacity at operating pH, and that proteins are cleaved by liberating amino- and carboxyl groups, which both undergo dissociation leading to equilibria between their acid- and base forms. The balance of hydronium ions produced per fraction of peptide bonds cleaved and referred to the initial concentration of peptide bonds leads to [5]:

$$x_H = \frac{1}{c_0}\frac{d[H_3O^+]}{dX} = x_{N,b} - x_{C,a} \quad (1)$$

with:

$$x_{N,b} = \frac{1}{1+10^{(pK_{a,N}-pH)}} \text{ and } x_{C,a} = \frac{1}{1+10^{(pH-pK_{a,C})}}$$

since hydronium ions are only created by appearance of the deprotonated amino group (base form), and hydronium ions are consumed by generation of the protonated carboxyl group (acid form). The degree of hydrolysis (X) should not be confused with the fraction of soluble protein (Y). The same may be added for the initial concentration of peptide bonds (c_0) and the initial protein concentration (S_0). The buffering capacity of the reaction system is described by the buffering capacity of those dissociating groups liberated by proteolysis. Thus, the buffering capacity of the reaction system, neglecting the dissociation of water, may be written as [8]:

$$\beta = -\frac{d[H_3O^+]}{dpH} = \ln(10)[c_0 X(x_{N,a}x_{N,b} + x_{C,a}x_{C,b})] \quad (2)$$

Combining equations 1 and 2 leads to the following:

$$\int_{pH_0}^{pH_f} dpH = -c_0 \int_{X_0}^{X_f} \frac{x_H}{\beta} dX \quad (3)$$

from which the pH profile as a function of the fraction of peptide bonds cleaved may be obtained by numerical integration. If characteristic parameters for the actual reaction system are used [5], the pH profiles shown in Fig. 2 are obtained. The initial concentration of peptide bonds (c_0) has been estimated to correspond approximately to the actual reaction conditions. According to the assumptions applied, extremely steep

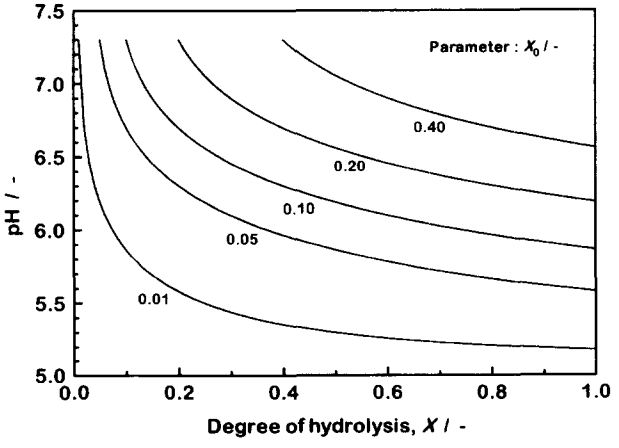

Fig. 2. Simulation of the pH shift due to protein hydrolysis at various initial degrees of hydrolysis. (Parameters: $pK_{a,N}$ = 7·0; $pK_{a,C}$ = 3·1; c_0 = 0·4 mol l^{-1})

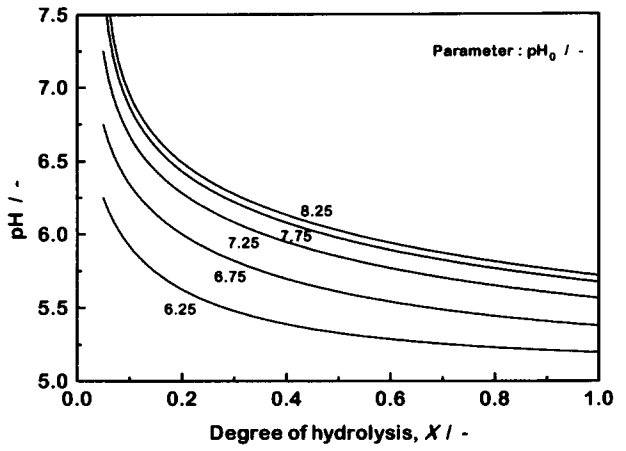

Fig. 3. Simulation of the pH shift due to protein hydrolysis at various initial pH. (Parameters: $pK_{a,N}$ = 7·0; $pK_{a,C}$ = 3·1; c_0 = 0·4 mol l^{-1})

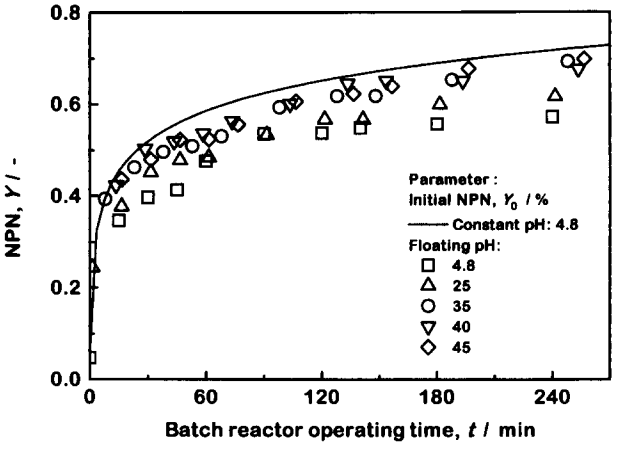

Fig. 4. Batch reactor performance at floating pH and varied initial fractions of soluble protein (T_c = 55°C; pH_0 = 7·3). The solid line shows the performance at a constant pH of 7·3 for comparison [9].

pH profiles may be obtained in the absence of hydrolysis products. A pre-hydrolysis of 5% would still lead to a pH drop of the order of 1·5. Higher degrees of pre-hydrolysis, however, would alleviate the problem of pH stabilisation more and more, because a small drop in pH would have a negligible effect on enzymic productivity. The simulation given in Fig. 3 shows that an initial pH higher than 8 would have no appreciable effect on stabilising the operating pH.

Batch reaction at floating pH

The influence of operating at floating pH was assessed by studying the reaction system in a stirred batch reactor. These experiments were performed by starting the reaction under pH control and switching to floating-pH operation when a predetermined fraction of soluble protein (NPN) was attained. The reaction was followed by analysing NPN as a function of the operating time. Data for operation at 55°C are shown in Fig. 4, in which the results for operation at floating pH are compared with those obtained under pH control at pH 7·3 taken from a previous publication [9]. As expected, the performance of a batch reactor operated at constant pH was only approached, when floating-pH operation was switched on at high degrees of pre-hydrolysis. A similar series of experiments was performed at an operating temperature of 60°C (Fig. 5). Switching to floating pH affected the hydrolysis at lower operating times, but was beneficial at higher operating times, particularly, when the operating mode was switched at high degrees of conversion. Thus, operation at floating pH apparently reduced deactivation by autodigestion, if the drop of pH was moderate. Higher operating temperatures are always of considerable interest owing to lowered risk of microbial infection. The experimental pH profiles as a function of batch reactor operating time are shown in Fig. 6 for the series of experiments at 55°C. These profiles compare well with the simulated profiles discussed before based on a rather simple model. When the pH drop at an operating time of 4 h in a batch reactor was taken as a characteristic quantity the correlations shown in Fig. 7 were observed. A pH drop of less then one unit may only be obtained in a tubular reactor or a batch reactor starting with an initial NPN in excess of 35%.

Performance of a CSTR

This pH characteristic of the reaction system made it unattractive to operate a single tubular reactor in which pH control would not be possible. In contrast a continuous stirred tank reactor (CSTR) may be operated easily with pH control, but would show lower productivity due to backmixing. The kinetics of the

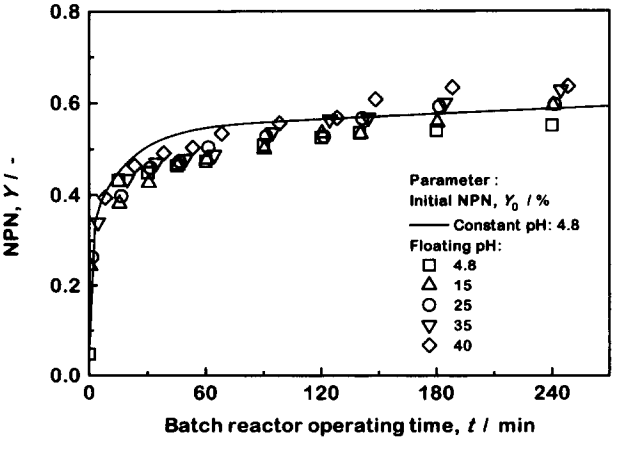

Fig. 5. Batch reactor performance at floating pH and various initial fractions of soluble protein (T_c = 60°C; pH_0 = 7·3). The solid line shows the performance at a constant pH of 7·3 for comparison [9].

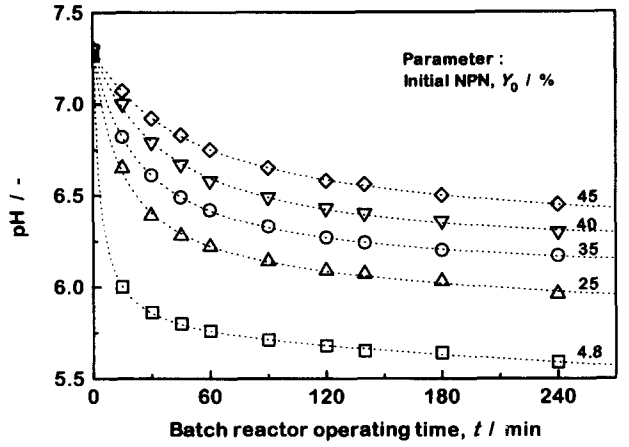

Fig. 6. Profile of pH in a batch reactor operating at varied initial fractions of soluble protein. (T_c = 55°C; pH_0 = 7·3).

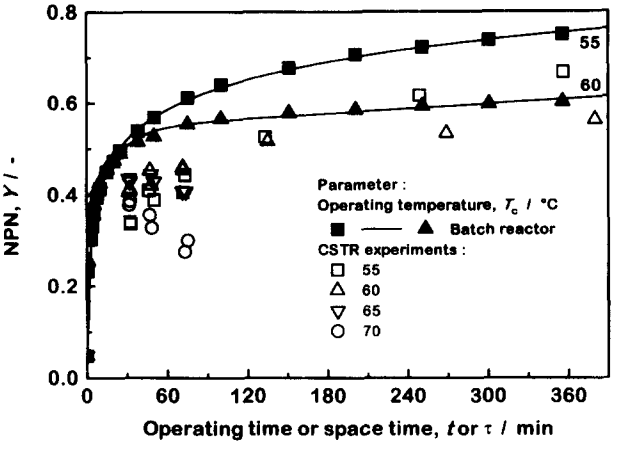

Fig. 8. Performance of a continuous stirred tank reactor (CSTR) operated at pH 7·3 at varied operating temperatures. The solid lines and symbols show the performance of batch reactors at a constant pH of 7·3 for comparison [9].

reaction system, characterized by high initial rates, but severe product inhibition [9], suggested that reasonable productivities may still be obtained even in a CSTR operating at a moderate NPN and therefore a low residence time. In Fig. 8 NPN profiles as a function of residence time in a CSTR operated at different temperatures are given in comparison with profiles known from stirred-batch reactors [9]. As expected, the CSTR operation always led to considerably lower yields of soluble protein at the same operating times. The decline in NPN with increasing time observed for experiments at 70°C was due to a non-trivial deactivation mechanism in combination with the balance of a CSTR [10, 11]. For practical reasons, an operating temperature of 55°C would be adequate for operating a CSTR at low residence times. The operation of a CSTR at 55°C, but at a different operating pH gave the results shown in Fig. 9. As anticipated, a better performance was obtained when operating at a pH of 7·8 instead of lower values.

Simulation of a staged reactor

For continuous reactor operation, a pH-controlled CSTR was used as a first stage and high yields of soluble protein were obtained by combining the CSTR with a tubular reactor (TR) operating at long residence times under floating pH. Continuous reactor operation requires considerable amounts of substrate and enzyme. However, such a reactor combination may be simulated by operating a CSTR, taking the partially converted reaction mixture at the reactor exit and treating it once again for a given time at the desired operating temperature without pH control in a reactor with negligible backmixing. Thus, the tubular part of the reactor was simply simulated by means of a batch reactor. Experimental simulation for both operating temperatures, 55 and 60°C were performed and the results are shown in Fig. 10. For isothermal operation,

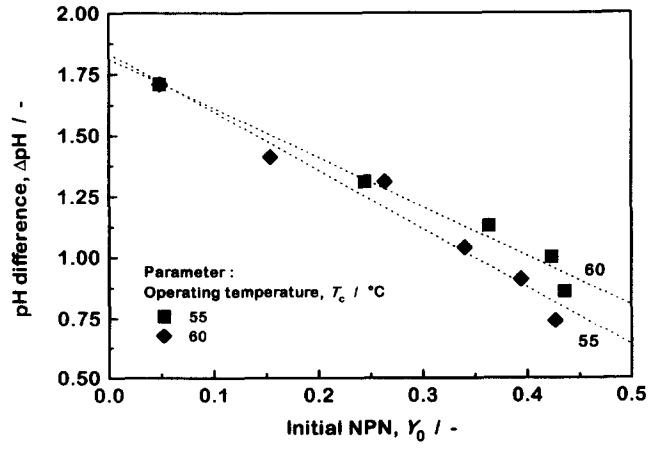

Fig. 7. Drop of pH for batch reactor operation at floating pH.

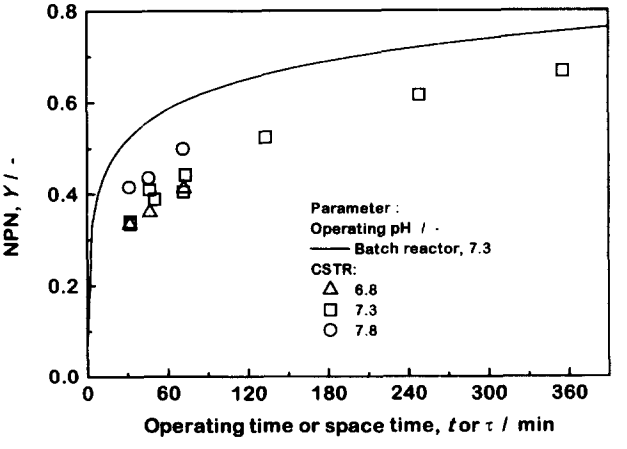

Fig. 9. Performance of a continuously operated stirred tank reactor (CSTR) operated at 55°C and varied pH. The solid line shows the performance of a batch reactor at a constant pH of 7·3 for comparison [9].

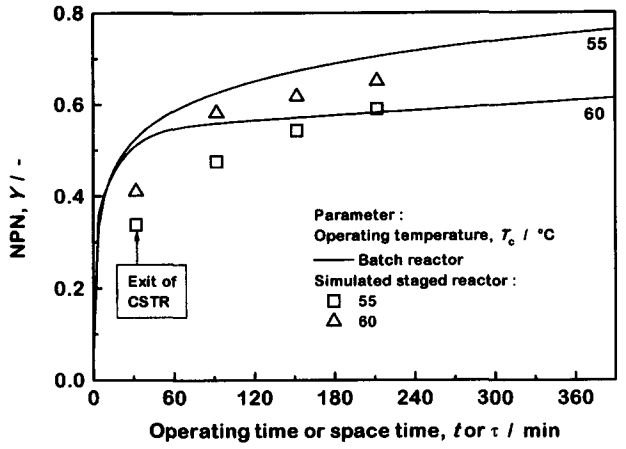

Fig. 10. Performance of an experimentally simulated staged reactor operated at 55 and 60°C, with identical temperatures in both stages, stirred tank and simulated tubular device. The pH was kept at 7·3 in the CSTR. The solid lines show the performance of batch reactors at a constant pH of 7·3 for comparison [9].

an operating temperature of 60°C was preferred and, knowing the influence of the operating pH on the performance of the CSTR, a series of experiments was performed by changing both the operating temperature and the pH of the CSTR. The tubular reactor was simulated as discussed above by treating the reaction mixture in a batch reactor for a given time at 60°C. The results are given in Fig. 11 and clearly show that maintaining the CSTR at 55°C and a pH of 7·8 was optimal for obtaining high yields of soluble protein for reasonable residence times in the simulated tubular device kept at 60°C.

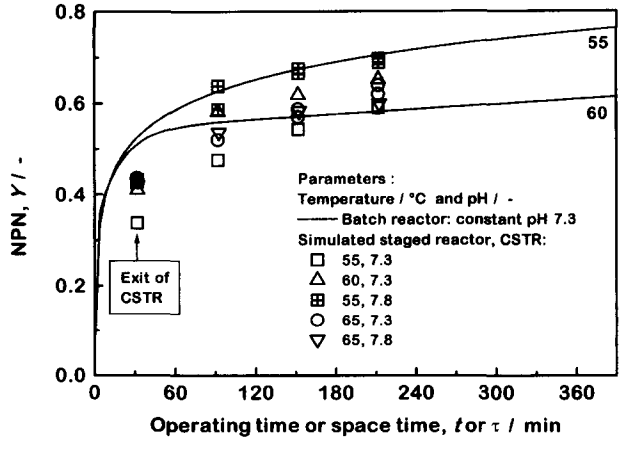

Fig. 11. Performance of an experimentally simulated staged reactor. The CSTR was kept at various temperatures and pH values, whilst the tubular reactor was simulated for an operating temperature of 60°C. The solid lines show the performance of batch reactors at a constant pH of 7·3 for comparison [9].

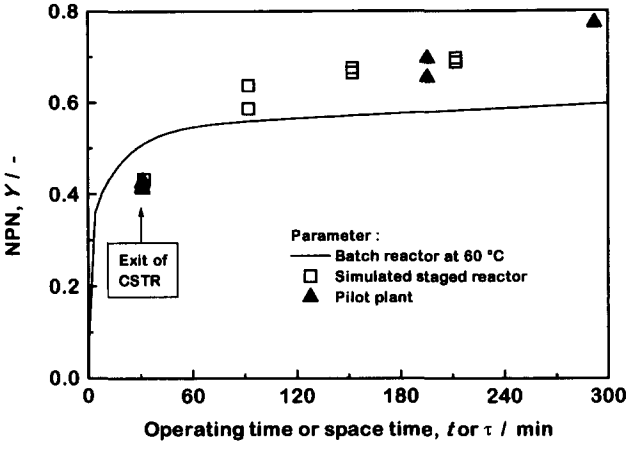

Fig. 12. Performance of the pilot-plant reactor, the stirred tank of which was operated at a constant pH of 7·8 and 55°C, whilst the tubular device was kept at 60°C. For comparison data are given for a simulated staged reactor operated under identical conditions and a batch reactor operated at a constant pH of 7·3 [9].

Pilot-plant studies

The pilot-plant consisting of a stirred tank and a tubular device (Fig. 1) was operated under the conditions outlined above. The CSTR was kept at a residence time of 30 min at 55°C and pH 7·8, whereas the residence time in the tubular part of the reactor was changed by altering the length of the tube. The results obtained from the pilot-plant are given in Fig. 12 together with the results of the simulated staged reactor under the same operating conditions. As expected from the use of static mixers as internals, the tubular reactor behaved like a plug-flow reactor.

Conclusion

Analysis of the limited hydrolysis of whey protein by soluble trypsin has led to the development of a continuously operated reactor comprising a stirred tank in series with a tubular device. The stirred tank allowed for the control of pH by titrating most of the hydronium ions produced by the reaction and maintaining the pH in the optimum range for trypsin activity. The tubular device equipped with static mixers behaved like an ideal tubular reactor and was operated at high residence times, in order to obtain a fraction of soluble protein, which was required to exceed 60%. This has been achieved by combining these two reaction devices with opposite behaviour with respect to backmixing [12].

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